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VACUOLAR ACIDIFICATION: MORE THAN THE SUM OF ITS PARTS?

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Abstract

Petunia flower colour is dependent on vacuolar pH and is therefore used to study acidification mechanisms. Recently, it was shown that the concerted action of two tonoplast-localised P_3-ATPases is required to hyperacidify vacuoles of petunia petals. Here we discuss how steep cross-tonoplast pH gradients may be established in specific cells.

The acidity of plant vacuoles varies between plant species, organs and cell types. In morning glory (*Hypomea tricolor*), when the slightly acidic vacuoles of flowers become neutral, a shift from a purple to the characteristic blue flower colour can be observed. By contrast, in CAM plants or fruits such as lemon (*Citrus)* or non-ripe grape berries (*Vitis*) the vacuolar pH can reach values as low as 2 or 3. Two types of proton pumps, a H^+-ATPase and a H^+-PPase have originally been shown to reside at the vacuolar membrane [1]. Besides acidification of the vacuolar lumen, proton pumping also generates a transmembrane gradient in electric potential, ΔE_m. Both gradients, ΔpH and the ΔE_m, are exploited to accumulate solutes within the vacuole. While ΔE_m regulates ion channels that mediate vacuolar uptake of anions such as chloride and malate, ΔpH drives cation/proton, anion/ proton and sugar/proton antiporters [2]. Until recently it was thought that V-ATPases and V-PPases at the tonoplast are exclusively responsible for generating the vacuolar pH. However, recent studies comparing the pH of a vacuolar V-ATPase double mutant (*vha-a2/vha-a3*) with that of a V-PPase mutant suggested that yet another component was involved in the generation of vacuolar pH. Possibly, the activity of the V-ATPase residing in the trans-Golgi network/ early endosomes also contributes to the generation of vacuolar pH [3]. While the V-PPase is likely to be important for vacuolar acidification during embryo development and the early stage of seedling formation, its major role in adult plants is the hydrolyzation of PPi to reduce PPi contents and support gluconeogenesis [4].

Petunia flowers can be used to study vacuolar acidification processes

Petunia (*Petunia hybrid*a) flowers are an ideal model system to study vacuolar pH and the genes involved in its generation. Anthocyanins accumulate in the vacuole

of flower petal cells, and the colour of these anthocyanins is dependent on vacuolar pH, amongst others. Akin to morning glory flowers, in petunia a shift in vacuolar pH causes a change in flower colour. Mutants that display a flower colour different to the red-flowering wild type are a useful tool to analyse gene function in vacuolar pH maintenance. Overall, seven pH-mutants defective in petunia flower colour and pH have been identified and termed *ph1* to *ph7* [5].

Within the last decade the team around Amsterdam's VU University researcher Francesca Quattrocchio has been successful in identifying several genes that are affected in the respective mutants, using transposon-tagging strategies. Some of the genes such as *PH3*, *PH4* and *PH6* are transcriptional regulators. In 2008 the team identified the mutant *ph5* to be defective in a gene encoding a proton pump [5]. PH5 localises to the tonoplast and belongs to the H^+-P_{3A}-ATPase subfamily, which finds homologs in the AHA family of *Arabidopsis* plasma membrane H^+-ATPases. Three lines of evidence led the researchers to conclude that PH5 is responsible for the transport of protons into the vacuole: first, PH5 expression was able to rescue a yeast mutant unable to grow on acidic medium; second, the difference in flower colour in *ph5* mutants was not caused by altered anthocyanin accumulation; and third, over-expression of a PH5 transgene in a *ph5* mutant background rescued the mutants' petal pH and colour phenotype. Interestingly, PH5 overexpression in the background of other pH-mutants such as those of the transcriptional regulators *ph3*, *ph4* and *ph6* could not rescue the pH and flower colour phenotype of those mutants. Seeing that *PH5* expression is regulated by *PH3*, *PH4* and *PH6*, the authors reasoned that these regulators may control yet another component, which, aside PH5, was necessary for vacuolar acidification.

The P_{3B}-ATPase, PH1, is the second component required to rescue petunia pH-mutants

In a recent study the team reports the identification of PH1 as an interactor of PH5, necessary for hyperacidification of petal vacuoles [6]. They obtained a transposon-tagged *ph1* mutant line and were able to identify the gene-sequence associated with *ph1* to code for a P_{3B}-ATPase.

Evidence that PH1 was the missing component in PH5-driven vacuolar acidification came from gene-expression analysis. *PH1* mRNA expression paralleled that of *PH5* spatio-temporally. Like *PH5*, mRNA expression of *PH1* was under the control of the transcription factors *PH3* and *PH4* and the transcriptional regulators *PH6* and *AN1*. While a *35S*-driven *PH5*-GFP construct localised to the tonoplast in the petal epidermis of petunia, only an internally GFP-tagged *35S*:*PH1-GFPi* construct localised to the tonoplast.

In light of the facts that PH1 and PH5 both encode P_3-ATPases, are both under parallel transcriptional control and overlap in their intracellular localisation, the researchers hypothesised that PH1 and PH5 acted in concert in vacuolar petal acidification. In fact, when overexpressed on their own, neither PH5, nor PH1 could rescue the colour and pH phenotype of the *ph3* mutant. Only when PH1 and PH5 were co-expressed the mutant's flower colour reverted back to red, and its petal extract pH decreased from ca. 5.7 to 5.3 (Figure 1). An elegant way to show that this mutant rescue was due to the acidification of petal cell vacuoles was the authors' co-expression of the Na^+/ H^+ and K^+/ H^+ antiporter NHX1, which had been shown to abolish cross-tonoplast pH gradients [7]. The PH1/PH5-mediated rescue of the *ph3* mutant colour and petal extract pH was lost when NHX1 was expressed in addition:

the flower colour reverted back to the pale-rosé of the *ph3* mutant, even though PH1 and PH5 were expressed.

Hyperacidification of petunia petal cell vacuoles relies on both P_3-ATPases

The final and crucial question addressed was: What constituted the concerted action of PH1 and PH5 in vacuolar acidification on a mechanistic level? To answer this questions the team carried-out interaction studies using Bimolecular Fluorescence Complementation as well as split-ubiquitin-system assays. Both approaches yielded that PH1 and PH5 were able to interact with one another. Further, they applied the patch-clamp technique to whole-vacuoles of petunia leaf cells. Clamping the membrane potential at 0 mV the team could observe ATP-dependent outward currents, arising from proton-transport from the cytosol to the vacuolar lumen. The vast majority of this current was sensitive to bafilomycin, an inhibitor of V-ATPases. However, in vacuoles expressing PH5, about half of the ATP-dependent current was sensitive to bafilomycin while the other half was sensitive to vanadate, an inhibitor of P-ATPases such as PH5. This indicated that PH5 was transporting protons to the vacuolar lumen. Surprisingly, vacuoles expressing PH1 did not display any vanadate-sensitive current, but when PH5 and PH1 were co-expressed the vanadate-sensitive current nearly doubled, when compared to vacuoles expressing PH5 only. This indicates that, while PH1 does not seem to have proton-transport activity, it enhances the PH5-mediated current. How such an enhancement may work on a mechanistic level, however, remains elusive.

PH1 displays sequence similarity to bacterial P_{3B}-ATPases such as MgtA and MgtB, which are required for Mg^{2+} uptake. Such transporter systems had previously been thought absent in plants but when the researchers carried out phylogenetic

analysis they found homologues in other species including *Vitis vinifera*, the berries of which also require vacuolar hyperacidification. Although the authors hypothesised that PH1 might boost PH5 H^+ pumping by dissipating ΔE_m through Mg^{2+} import to the cytosol, they found no evidence for this and reasoned that such an import was energetically unfavourable.

Concluding remarks and outlook

The authors of this review found that, uniquely, PH1 is lacking an aspartate (D) residue that is conserved in Mg^{2+}-, Ca^{2+}-, Na^+/K^+-, H^+/K^+-, and H^+-ATPases such as the prokaryotic PH1-homologues and PtPH5, and which has been proposed to be essential for cation binding and translocation [8]. In PhPH1 this residue is replaced by an asparagin (N). When the corresponding Asp 684 of the Arabidopsis H^+-ATPase AHA2 was mutated to Asn (D684N), the enzyme still displayed ATP hydrolysis activity but did no longer show coupling to H^+ transport [8]. The coupling ratio of V-ATPases depends on vacuolar pH, pumping $4H^+$ to $2H^+$ per ATP as ΔpH increases [9]. H^+-P-ATPases are essential for the energisation of even steeper gradients as they operate at coupling ratios of $1H^+$/ATP [10]. Taken together, we speculate that when PH1 and PH5 interact, this ratio is decreased even further to hypothetically $0.5H^+$/ATP allowing for hyperacidification. In order to test this hypothesis, initially one may generate the mutation N782D in PhPH1 in order to determine if proton-pumping activity is restored and the resulting pH of two pumps working in parallel could be determined. Furthermore, kinetic approaches using the patch-clamp technique may be employed to investigate the H^+/ATP coupling ratio of the interacting PH1-PH5.

In conclusion, the study described above has not only identified a missing component in petunia flower colour and pH regulation, but it may point to an entirely new mechanism of proton-pumping via P-ATPases.

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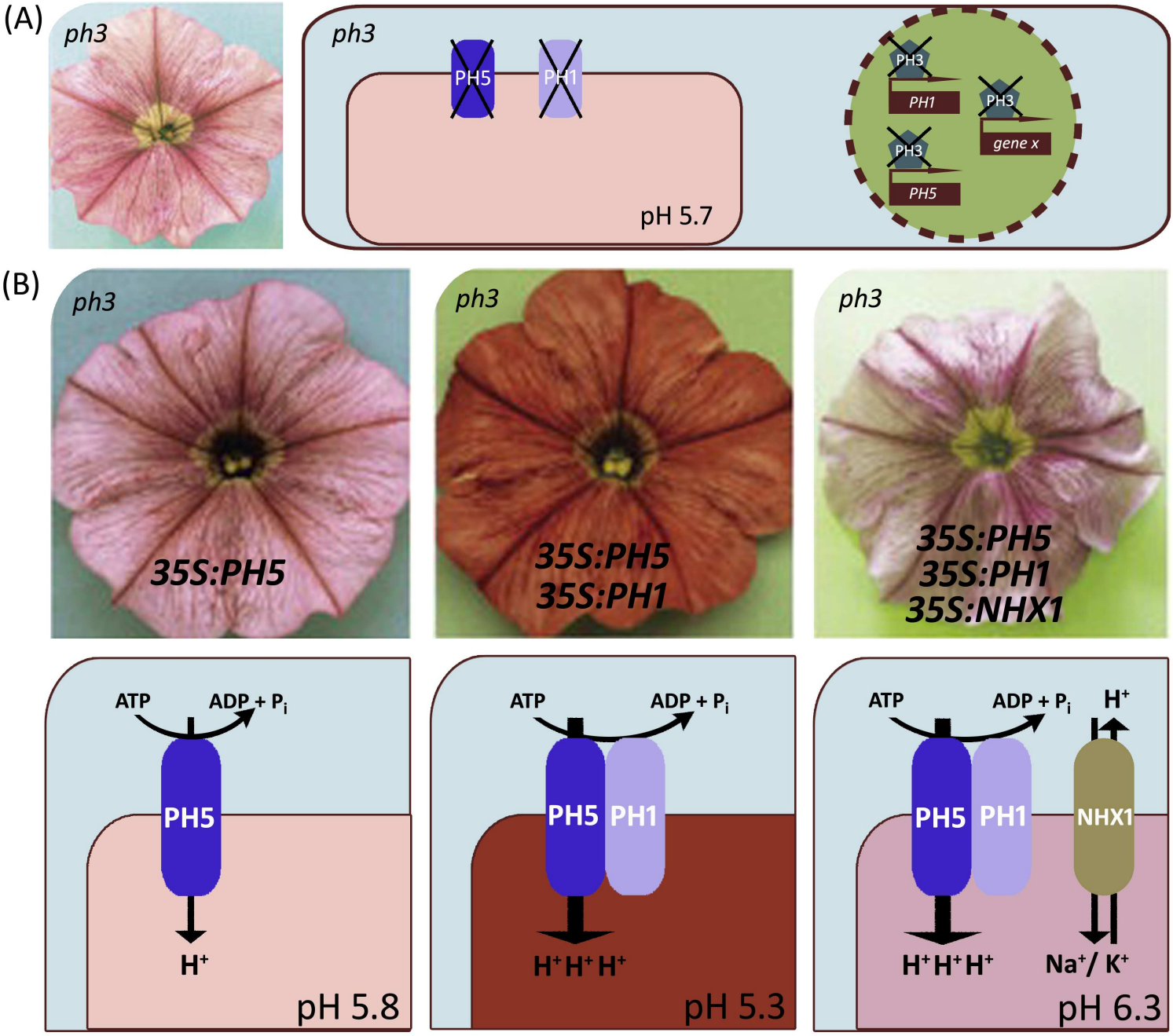
Figure legend

Figure 1. Petunia flower colour and pH phenotype of the *ph3* mutant is restored by the constitutive co-expression of the P-ATPases PH1 and PH5.

(A) In the *ph3* mutant PH3-regulated genes such as *PH1* and *PH5* are not expressed, resulting in a pale-rosé flower phenotype and an increase in vacuolar pH compared to

wild-type petunia flowers. (B) The flower and vacuolar pH phenotype of *ph3* mutants is only rescued when 35S:PH1 and 35S:PH5 are co-expressed. The additional introduction of the Na^+/ H^+ and K^+/ H^+ antiporter NHX1 restores the *ph3* mutant phenotype. Reproduced, with permission, from [6]. (C) Multiple protein sequence alignment of the putative transmembrane domain 5 and 6 of *At*AHA2 according to the uniprot database including the conserved aspartate residue (D684 in *At*AHA2) between the *Arabidopsis thaliana* AHA2 and AHA10 (homolog of *Ph*PH5), PH5 and PH1 of *Petunia hybrida*, and the bacterial P_{3B}-ATPases, MgtA and MgtB of *Salmonella typhimurium*. Asterices indicate sequence identity, dots the degree of similarity. The alignment was performed using ClustalW.


(A)
ph3
ph3
PH5
PH1
pH 5.7
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PH1
PH3
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PH5
(B)
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35S:PH5
ph3
35S:PH5
35S:PH1
ph3
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35S:PH1
35S:NHX1
ATP
ADP + Pi
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ADP + Pi
PH5
PH1
H+H+H+
pH 5.3
ATP
ADP + Pi
H+
PH5
PH1
NHX1
H+H+H+
Na+/ K+
pH 6.3
(C)
TM5
TM6
AtAHA2 639 FQRMKNYTIYAVSITIRIVFGFMLIALIWEFD-FSAFMVLIIAILNDGTIMTISKDRVK-- 696
AtAHA10 646 FQRMRNYTVYAVSITIRIVLGFTLLALIWEYD-FPPFMVLIIAILNDGTIMTISKDRVR-- 703
PhPH5 646 FQRMKNYTIYAVSITIRIVLGFMLLALIWKYD-FPPFMVLIIAILNDGTIMTISKDRVK-- 703
PhPH1 736 FGNTMKYIKMSVIANLGSIISLLIATLIFGFEPLTPMQLLTQNILYNLGQIAIPWDKMEDC 796
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StMgtB 701 FGNIIKYLNMTASSNFGNVFSVLVASAFIPFLPMLAIHLLIQNLMYDISQLSLPWDKMDKE 761
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